Adenine binding sites of F_1-ATPase are located on the surface of the protein molecule

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1. INTRODUCTION

Besides the active (catalytic) site(s), F_1-ATPase has several other nucleotide binding sites (see [1–3]). These sites differ in the mode of the nucleotide binding. Several sites bind the nucleotides tightly (nucleotides remain bound to F_1-ATPase after gel filtration or repeated precipitation by ammonium sulphate). F_1-ATPase cross-linked with dimethylsuberimidate can tightly bind 5 ADP molecules. This F_1-ATPase preparation containing tightly bound nucleotides exhibited ATPase activity [4]. In F_1-ATPase there is also the site (other than the active site), which exchanged bound nucleotides for the nucleotides of the medium at a high rate [3,5,6]. It is possible that this site, which is referred to as the non-catalytic in this article, may either reversibly or tightly bind nucleotides, depending on the ATPase conformation.

It has been shown that the active site of mitochondrial ATPase is localised on the β-subunit [7–12]. The results that we obtained using a 2′,3′-dialdehyde ADP derivative indicate that the non-catalytic site of F_1-ATPase is localised on the α-subunit [13]. In this study we have investigated to what extent $1,N^6$-etheno-adenine nucleotides (bound to the catalytic site, the non-catalytic site and to the sites of tight binding) is accessible to iodide, a hydrophilic fluorescence quencher. It is shown that the $1,N^6$-etheno-adenine moiety of the nucleotide bound to all 3 types of nucleotide binding site is accessible to iodide. Thus, in all 3 cases, the regions of F_1-ATPase that bind the adenine moiety of nucleotides are localised on the surface of the protein molecule.

Abbreviations: ϵ-ATP, $1,N^6$-etheno-ATP; ϵ-ADP, $1,N^6$-etheno-ADP; ϵ-ATPMC, mixed anhydride of $1,N^6$-etheno-ATP and mesitylene carboxylate; oxϵ-ADP, 2′,3′-dialdehyde derivative of $1,N^6$-ethano-ADP

2. MATERIALS AND METHODS

F_1-ATPase was obtained from beef heart mitochondria according to [14]. The mixed anhydride of $1,N^6$-etheno-ATP and mesitylene carboxylate (ϵ-ATPMC) was synthesized by Dr N.I. Sokolova and Professor Z.A. Shabarova. The 2′,3′-dialdehyde $1,N^6$-etheno-ADP derivative (oxϵADP) was obtained from ϵ-ADP (Serva) by periodate oxidation, according to [15]. For the fluorescence quenching highly purified KI (Reachim) was used.

Treatment of F_1-ATPase (2–2.5 mg/ml) with 2 mM ϵ-ATPMC was carried out for 1 h in 100 mM Tris–HCl, 0.1 mM EDTA (pH 7.5). For the separation of the modified enzyme from the free nucleotide a column of Sephadex G-50 fine (1 × 25 cm) equilibrated with 10 mM Tris–HCl (pH 7.5) was used. The fluorescence measurements of the modified enzyme were made on a Hitachi EPC-3 spectrofluorimeter. The wavelength of the exciting light was 320 nm. To determine the

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degree of modification of F_1-ATPase by ϵ-ATPMC, the intensity of the fluorescence of the modified enzyme at 420 nm was measured (see [16]). The stoichiometry of F_1-ATPase/ϵ-ATPMC was 1:1.

The treatment of F_1-ATPase with oxϵ-ADP was done as in [13]. The enzyme was desalted using a G-25 Sephadex column (1 × 25 cm), equilibrated with 30 mM morpholinopropane sulphonate buffer, 2 mM EDTA (pH 8.5). The protein solution (1 mg/ml) was incubated with 0.3 mM oxϵ-ADP for 1.5 h. Two additions of sodium borohydride with a 15 min interval were made, up to 2 mM final conc. The protein was precipitated by ammonium sulphate and dissolved in 10 mM Tris–HCl (pH 7.5). Free oxϵ-ADP was removed by gel filtration on a Sephadex G-50 fine column.

Tight ϵ-ADP- and ϵ-ATP-binding to F_1-ATPase was studied as in [18]: F_1-ATPase (2–2.5 ml) was incubated for 30 min in 100 mM Hepes buffer (pH 6.7), 0.25 sucrose, and 3 mM ϵ-ADP or ϵ-ATP. F_1-ATPase containing tightly bound nucleotides was separated from free ϵ-ADP or ϵ-ATP, using a column of Sephadex G-50 fine (10 mM Tris–HCl, pH 7.5). The stoichiometry of nucleotide binding was determined by measuring ϵ-ADP or ϵ-ATP fluorescence after the release of tightly bound nucleotides in the presence of sodium dodecyl sulphate.

3. RESULTS AND DISCUSSION

The accessibility of the nucleotide binding sites of ATPase to hydrophilic ions was studied by measuring the fluorescence quenching of bound nucleotides by KI. Quenching of the fluorescence of etheno-adenine nucleotide by KI is the result of the direct contact of iodide ions with the etheno-adenine moiety of the nucleotide molecule [19,20].

Fig. 1 (curve 1) shows the dependence of the fluorescence intensity of free ϵ-ADP on the KI concentration. A similar picture was obtained for free ϵ-ATP (not shown).

Fig. 1 (curve 2) shows the quenching of the fluorescence of etheno-adenine covalently bound to the active site of F_1-ATPase as a result of the treatment of F_1-ATPase with ϵ-ATPMC. From the results obtained it follows that the adenine moiety of the nucleotide bound to the ATPase active site is only partially (apparently from one side) shielded by the protein molecule from the hydrophilic

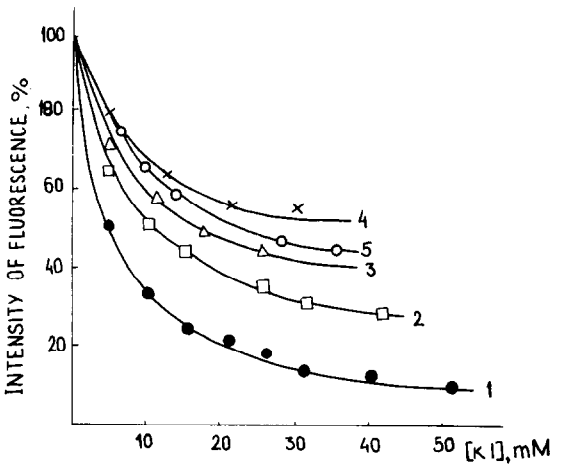

Fig. 1. Fluorescence quenching of free and F_1-ATPase-bound etheno nucleotides by KI: curve 1, free ADP (8×10^{-7} M); curve 2, F_1-ATPase (5×10^{-7} M) treated with ϵ-ATPMC; curve 3, F_1-ATPase (2×10^{-7} M) treated with oxϵ-ADP; curve 4, F_1-ATPase (9×10^{-7} M) containing tightly bound ADP (2.7×10^{-7} M); curve 5, F_1-ATPase (1.6×10^{-6} M) containing tightly bound ATP (8×10^{-7} M). The measurements were made in 10 mM Tris–HCl buffer (pH 7.5).

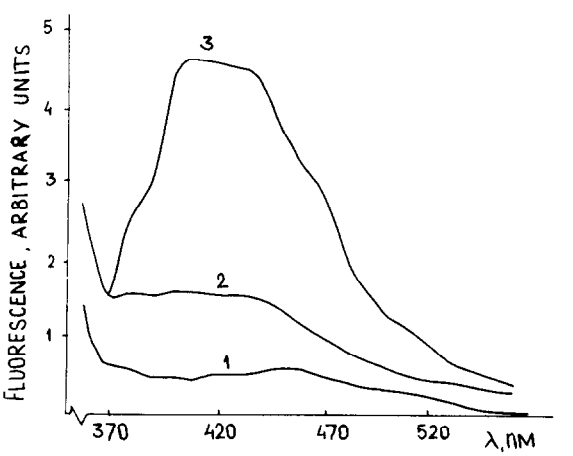

Fig. 2. The fluorescence spectrum of F_1-ATPase treated with oxϵ-ADP: curve 1, 10 mM Tris–HCl (pH 7.5); curve 2, 10 mM Tris–HCl (pH 7.5), standard preparation of F_1-ATPase (3×10^{-7} M); curve 3, 10 mM Tris–HCl (pH 7.5) and 3×10^{-7} mM F_1-ATPase treated with oxϵADP. The wavelength of the exciting light was 320 nm.

iodide ions. Such a situation may occur when the region of the adenine binding is located on the surface of the protein molecule in contact with water.

As shown elsewhere, oxADP selectively modifies the non-catalytic nucleotide binding site of ATPase [13]. Here we studied the reaction of F_1-ATPase treated with oxϵ-ADP. Fig. 2 (curve 3) shows the fluorescence spectrum of F_1-ATPase treated with oxϵ-ADP. The stoichiometry F_1-ATPase/oxϵ-ADP calculated from the data of fig. 2 is equal to 0.9–1.0. Similar data on stoichiometry were obtained in [13], when studying the reaction of F_1-ATPase with [^{3}H]oxADP. F_1-ATPase treated with oxϵ-ADP (as well as F_1-ATPase treated with oxADP [13]) exhibited full ATPase activity.

Fig. 1 (curve 3) shows the quenching of the fluorescence of etheno-adenine bound to the non-catalytic site of F_1-ATPase as a result of treatment of the enzyme with oxϵ-ADP. As in the case of the active site, the etheno-adenine bound to the non-catalytic site of F_1-ATPase is partially accessible to the iodide ions. This result testifies to the fact that the region of the adenine binding in the non-catalytic site is located on the surface of the protein molecule in direct contact with water.

We also studied the location of the tight nucleotide binding sites of F_1-ATPase. According to [2,18], the sites of tight nucleotide binding are highly specific, thereby ϵ-ADP and ϵ-ATP bind to these sites much less easily than ADP and ATP. The results obtained in this work show that incubation of F_1-ATPase with 3 mM ϵ-ADP or ϵ-ATP at pH 6.7 (see section 2) leads to the tight binding of 0.3–0.5 mol nucleotides/mol enzyme. Fig. 1 shows the fluorescence quenching of tightly bound ADP (curve 4) and tightly bound ϵ-ATP (curve 5) by KI. Control experiments (not shown) indicated that 40 mM KI does not lead to the release of tightly bound nucleotides from F_1-ATPase. From the results obtained it follows that the etheno-adenine moiety of the tightly bound nucleotides is partially accessible to iodide; i.e., the heterocyclic base of the tightly bound nocleotides is located on the surface of the F_1-ATPase molecule. This result should be regarded as unexpected. In fact, the heterocyclic base is the most hydrophobic moiety of the nucleotide molecule and might thus be assumed to become immersed in the hydrophobic pocket of the F_1 molecule as a result of tight nucleotide binding. The results obtained make us think that this is not the case. It is interesting to compare this result with our data on the enhancement of the fluorescence of 2′(3′)-*O*-trinitrophenyl ADP derivative as a result of tight binding of this nucleotide to F_1-ATPase [21]. This fluorescence enhancement testified to the fact that the trinitrophenyl residue of tightly bound nucleotides is located in the hydrophobic surrounding and thus the region of F_1-ATPase responsible for the ribose binding is located in the hydrophobic pocket of the protein molecule [21].

Thus, the tight binding of the nucleotides to F_1-ATPase is something of a paradox: the heterocyclic base binds to the surface of the enzyme, but the more hydrophilic ribose residue becomes immersed in the hydrophobic pocket of the protein molecule.

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